STUDIES ON SULPHUR BACTERIA IN THE PRAWN CULTURE ECOSYSTEM

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Shri S. ALAGU RAVI
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C E R T F I C A T E

This is to certify that this Dissertation is a bona-fide record work carried out by Shri.Alagu Ravi, S. under my supervision and that no part thereof has been presented before for any other degree.

V. Chandrika.

(Dr.V.Chandrika)
Scientist,
Central Marine Fisheries
Research Institute,
COCHIN.

Co intersigned by

Director,
Central Marine Fisheries
Research Institute,
COCHIN.

C O N T E N T S

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P R E F A C E

Aquaculture, in the warm waters of South-east Asia is acknowledged the world over to be several times more remunerative than agriculture. Prawn or shrimp culture and fin-fish culture in ponds, paddy fields, mangroves, salt pans, cages and pens, mussel culture on poles and ropes suspended from rafts and algal culture on ropes and nets are all being attempted now successfully in India on an experimental basis by the Central and State Government Fishery Organisations. There is a vast scope for extending these efforts into large commercial scales either by the government itself or by the private enterpreneurs thereby providing immense opportunities for rural employment.

Applied research on every culture ecosystem and on every culturable organism has to be pursued on a multi-disciplinary level but in a more integrated way, with the sole aim of establishing the technology for high yield farming of each species under Indian conditions. In Kerala though some semi-intensive cultures are done, mainly extensive culture has been practiced in pokkali fields. Compared to highly sophisticated culture operations in some of the developed countries, the traditional culture operations in natural ponds of Kerala depends almost entirely on the natural source of water for the successful culture operation.

It is a known fact that micro-organisms influence chemical, physico-chemical, geological and biological conditions of the ponds. In aquaculture, once an acceptable environment has been

found, maintaining the environmental quality must be considered as one of the most important challenges facing the aquaculturist. In this context, knowledge of bacteriology of the ponds is extremely important to aquaculturist. Nearly, every problem that arises in an aquaculture system is the result of interactions of microorganisms with environment.

Development of successful pond water management programme planning depends upon a complete knowledge of both the physical and biological processes working within a particular system. The turnover rates and exchange of nutrients with the sediments are in the part governed by biological communities. "Nutrients" refer to not only organic material, simple and complex, but also to trace elements, vitamins and also the major inorganic elements such as carbon, nitrogen, phosphorus and sulphur.

Sulphur in its various organic and inorganic forms is essential for all living organisms. The major reserve of the element in sediment is the organic fraction and the storehouse is only unlocked through biological decomposition, Microbial transformations of sulphur in the pond is governed to a large extent by the environmental circumstances that affect the composition and activity of the microflora. Whether one recognizes the fact or not, the natural ponds depends basically on two independent But not mutually exclusive, processes to cleanse its immediate environment: oxidation, both chemical and photo and biological oxidation or degradation.

Sulfate can be stoichiometrically reduced to hydrogen sulphide by degradative processes which, in turn, can be oxidized chemically in the presence of oxygen, to elemental sulfur. Elemental sulfur in turn can be oxidised to sulfate.

Also the production of hydrogen sulphide by a specific class of bacteria, the anaerobic dissimilatory sulfate reducers, leads to the stoichiometric production of hydrogen sulphide and consequently an anaerobic environment. On the other side the oxidation of elemental sulfur by 'Thiobacilli' leads to the production of sulfuric acid and their metabolic activity is evident in aquatic environment. Although not widely recognized, the availability of sulfur can limit the productivity of the culture ponds and has been linked to decreased productivity of fish.

Among th e nutrient recycling, sulphur cycle is from a geobiological point of view next in importance to photosynthesis and its potential milieu is essentially the same. (Bass, Becking, et.al, 1957). The cycling of each nutrient in the aquaculture ponds is inter-related in that any perturbation in one cycle has far reaching effects on the other cycles. For example, it has been shown that the sulfate reducing bacteria are capable, not only of nitrogen fixation, but degradation of carbon compounds to carbondioxide and also to effect a solubilization of phosphate as a consequence of precipitation of insoluble iron sulphides.

There are many examples of these inter-relationships in microbial communities and it is these relationships, these rates and these tranformations which require elucidation for proper water manage-

ment in aquaculture ponds.

Sulphur bacteria can also influence the pH, Eh, colour, carbonate content, oxygen tension and other properties of water or bottom mud and it renders mud and water uninhabitable by other organisms.

So considering all these points in the present study an attempt has been made to study the relation between the different groups of bacterial parameters concerned with sulphur cycle. Information on the physico-chemical parameters as well as their fluctuations in the culture ponds are equally important, since certain other parameters like temperature and hydrogen ion concentration have got synergistic effect in the distribution of sulfur bacteria when combined with other factors. In typical brackish water environment which is fed by tidal flow through the estuaries environmental changes occuring in the ecosystem over the period of time is of utmost importance while studying thionic micro-organisms present it. The samples were also analysed for physico-chemical parameters in two different prawn culture ponds (Perrennial pond 'A' and pokkali field, Pond 'B') for a period of 4 months from june to september 1984.

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I T R O D U C T I O N

Sulphur bacteria are the bacterial groups which oxidise or reduce sulphur or it's in organic compounds. These include sulfate reducers and all colourless forms which oxidise sulfur or its compounds as well as the purple sulfur bacteria and green sulphur bacteria (Zobell, 1946). In the culture point of view, production of hydrogen sulphide is of primary importance in culture ponds. Shigueno (1975) reported an amount of 9.975 ppm of sulphide from a culture pond which is proved to be lethal for shrimps by him. In this respect, sulphate reducers which play a main role in hydrogen sulphide prodáction is to be given primary impocrtance.

Sulphate reducing bacteria which belong to the genus Desulfovibrio and Desulfotomaculam(Postgate, 1979) have been given prime importance while studying sulphur cycle as they play key role in sulphur cycle (Zobell, 1946). Jorgensen(1977) found that sulphate reduction alone accounted for 53% of the total mineralisation of organic matter in brackish water sediment. It has been proved that sulphate reducing bacteria can cause phosphate release in mud as a consequence of precipitation of insoluble iron sulphide.(Baas Becking and Maccay, 1956). Herbert et al., (1977) reported nitrogen fixation by Desulfovibrio species.

A variety of organic and inorganic compounds in fresh, brakish and marine environments are reduced to hydrogen sulphide by sulphate reducing bacteria. They are found to utilise sulfite, thiosulphate, tetrathionate and metabisulphite (Postgate, 1951).

Zobell (1946) and Tezuka(1965) reported that they can utilise any kind of organic materials including proteins, sugars, starch, hydrocarbons, fatty acids, organic acid and alcohols. Werner Badziong et al., (1978) isolated a Desulfovibrio species which can utilise hydrogen and sulphate as it's sole energy sources. Abram and Nedwell (1979) have shown that hydrogen utilising sulphate reducing bacteria are capable of scavenging hydrogen and are the predominant hydrogen scavengers in the sediment of the Colene point salt marsh, U.K.

In pure cultures sulphate reducing bacteria have been found to utilise only a few compounds like pyruvate, lactate, succinate, malate and ethanol as their main carbon substrates.(Parkes and Poole, 1980; and Tezuka, 1966). Thus it has been thought they must rely on the metabolic end products from other heterotrophic bacteria which can metabolise more complex compounds (Tezuka, 1966; and Parkes and Poole, 1980). Tezuka (1966) demonstrated that a commensalism existed between sulphate reducing bacteria and other heterotrophic bacteria. The coexistence of two distinct functional groups of sulphate reducing bacteria in salt marsh sediments was proved by Ibrahim et al., (1981) and they also suggested a relationship between sulphate reducers and other organotrophs. The role of sulphate reducers in petroleum genesis has been discussed by many investigators (Jankowsky and Zobell, 1944; Tauson and Alioschina, 1932; Zobell 1946). The effect of sulphate reducers on iron corrosion is discussed by Starkey and Wight (1943); Starkey(1953).

Sulphate reducing bacteria play an impocrtant role in sediments by utilising sulphate as an electron accepter for

their metabolism with the concomitant production of hydrogen sulphide (Parkes and Poole, 1930). Baier(1935) proved that hydrogen sulphide was derived primarily from the bacterial reduction of sulphate and only to a slight extent from proteins. He also reported that the production of hydrogen sulphide corresponded to the periods during which there was much organic matter undergoing decomposition in the Little Kiel, Germany.

It is a known fact that hydrogen sulphide is toxic to organisms. There has been many reports on the mass mortality of aquatic animals due to hydrogen sulphide. Production of plankton and the following breakdown of organic matter and putrifaction of diatom and dinoflagellates in the sea bed can cause the production of hydrogen sulphide and the transportation of that bottom water rich in hydrogen sulphide can cause mortality of fishes and other organisms (Gunther, 1936; Brongersma-Sanders, 1948). Sudden death of fishes were recorded due to the occurrence of hydrogen sulphide in the surface waters (Brongersma-Sanders, 1957). Sebatiano Genovese (1963) reported that hydrogen sulphide cause a remarkable damage to the mussel farming in the Lake Fare. Shigueno(1975) in the Lab experiment found out that shrimps lost equilibrium when exposed to hydrogen sulphide of 0.1 ppm to 0.2ppm concentration and it succumbed to a concentration of 4.00ppm. Harmful effects of hydrogen sulphide to the marine, bottom invertebrates have been discussed by Theede et al., (1969).

Occurrence of hydrogen sulphide from different environments were reported by many workers. Copenhagen(1934) reported periodic production of hydrogen sulphide from bottom mud in quantities sufficient to be lethal to flora and fauna in the overlying water from Atlantic Ocean off Walvis Bay. According to Bunker(1936), Issatchenko reported the seasonal production of hydrogen sulphide in the sea of Azov in sufficient quantities to destroy large number of fishes and other organisms.

Production of hydrogen sulphide in estuarine environment through sulphate reduction has been reported by many investigators in various occasions. (Poole et al., 1977; Pengerud& Dundas, 1979; and Parkes et al.,1980).

Studies on sulphate reducing bacteria were started as early as in 1890s when Zelinsky (1893) reported Vibrio hydrosulfureus and Bacterium hydrosulfureum ponticum. Beijerinck (1895) described Spirillum desulfuricans and it was VanDelden (1904) who isolated a similar species Spirillum aestuarii in pure cultures. Taxonomy, physiology and biochemistry of sulphate reducers have been investigated intensively in the past. (Baars,1930; Starkey, 1938; Butlin etal., 1949; Postgate, 1951a; & 1951b; Grossman and Postgate, 1953; Taylor and Parkes, 1983). Excellent reviews were published by Postgate (1959)and Legall and Postgate(1973).

Sulphate reducing bacteria have been reported from different environments by several workers. However ecological aspects of sulphate reduction in natural environments like the distribu-

tion of sulphate reducing bacteria and their metabolic activity 'insitu' in relation to organic matter content of the medium, concentration of sulphate ion, accumulation of sulphides etc., have not not been widely investigated.(Tezuka,1979).

Beijerinck(1895) isolated Spirillum desulfuricans, a sulfate reducer from Delft ditch water. Ginsburg-Karagistscheva(1933) reported the occurance of sulphate reducers in produced waters from several oil wells. Young(1936) encountered sulphate reducing bacteria from shallow oil wells. Issatchenko(1914) noted the widespread occurence of Desulfovibrio aestuarii in Arctic sea bottoms. Issatchenko (1924) found sulphate reducing bacteria in bottom samples from the Black Sea. Bavendamm (1932) noted the presence of large numbers of sulfate reducers in calcarious mudaround the Bahama Islands causing precipitation of calcium carbonate under certain conditions. Copenhagen(1934) isolated sulphate reducing bacterium from black mud, off Walvis Bay and found that the mud contained no carbonate and came to a conclusion that sulfate reduction promotes the dissolution rather than the precipitation of calcium carbonate.

Zobell (1938a) demonstrated from 1000 to 10,000 sulphate reducers per gram of marine mud. Butkevich (1938) recorded sulphate reducing bacteria in the Caspian Sea. Zobell(1946) stated that the sulphate reducers were only rarely present in sea water except near the bottom. Wood (1959) isolated Desulfovibrio desulfuricans on three occasions during a six month study in the continental

waters of Australia. Marty(1981) reported the coexistence of sulphate reducing bacteria with three other groups of bacteria while studying the distribution of different anaerobic bacteria in Arabian sea sediments.

Sebastiano Genovese(1963) reported the presence of sulphate reducing bacteria from brakish water lakes. Wheatland(1954) investigated sulphide formation in the Thames estuary and found that the activity of sulphate reducing bacteria were less at low temperature of 5°C. Herbert et al.,(1977) while studying Nitrogen assimilation in marine environments reported that the dominant bacteria in sediments from all the sampling sites were sulphate reducing bacteria belonging to the genus Desulfovibrio in Kingoodie Bay sediments, Loch Eil, and Loch Etive, Scotland. Sulphate reducing bacteria have been reported from River Don Estuary, Scotland at various occasions. (Poole et al.,1979; Parkes and Poole, 1979). Tezuka(1979) isolated sulphate reducers from fresh, brakish and marine environments. Sethunathan et al., (1981) while studying pesticide metabolism in rice soil foundout thatsulphate reducing bacteria decreased due to the application of pesticides.

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MATERIALS AND METHODS

Study Area:

Studies on short term variations in sulphate reducers with respect to heterotrophic populations and some of the physico-chemical parameters were carried out.from two different prawn culture ecosystems at Narakkal ($10^{o}.01'N - 75^{o}.16'E$). For this purpose one perennial field (pond-A) from Prawn Culture Laboratory, Narakkal and a pokkali field (pond-B) from the same area were selected.

Pond-A

It is a brakish water perennial pond of area 0.6ha. The bottom nature of the sampling sites was sandy. The pond was connected to the main canal through a sluice gate. There was a constant exchange of water throughout the period of study influenced by tides.(Fig:1)

Pond-B

It is a pokkali field connected to the main cnal through similar type of fields. Water was stagnant and shallow throughout the period of study. Paddy crop was also under cultivation during the period of study.

Collection of samples.

Samples were collected forthightly from two fixed points from each pond. The surface water samples were collected

aseptically in sterilised 125 ml oxygen bottles for bacterial analysis. Water samples were collected separately in 200 ml plastic containers for chemical analysis. Mud samples were collected in polythene packets by using a plexiglass cover of the dimension 100 x 4 cm.

The samples were collected inbetween 08.00 hrs and 09·00hrs and were brought to the laboratory within two hours in an ice box. Samples were subjected to bacteriological analysis within three hours after the collection. Other chemical parameters of mud and water were foundout in the same day of collection. Samples were kept in a refrigerator at 4°C till the time of bacteriological analysis.

The following physico-chemical parameters were determined. Temperature was measured by using a mercury thermometer graduated into centigrades. Salinity of the water samples were estimated by using Mohr-Knudson, Argentometry. Dissolved oxygen content of the water samples were determined by Winkler's method. pH of mud and water samples were determined by an Elico pH meter using standard and calomel electrodes. Eh of water and mud samples were found out by the same pH meter by using a platinum electrode.

Isolation and quantitative analysis of bacteria:

Synthetic media from Hindustan dehydrated media (Hi-media) were used for the isolation, purification, maintenance and sub-culture of bacteria.

For total heterotrophic counts in mud and water Zobell's marine agar 2216 was used and pH was adjusted to 7.6. In the case of sulphate reducers also the same medium was used with the addition of separately sterilised 1% solution of lead acetate (40 ml/l) aseptically (Rodina, 1972). pH was adjusted to 7.5. pH of the media were adjusted by using N/10 N sodium hydroxide and N/10 hydrochloric acid solution.

For the quantitative enumeration of both sulphate reducers and total heterotrophs pour plate technique was used (Rodina, 1972). Serial dilutions of water and sediments samples were prepared using standard procedures given by Rodina (1972). Aged water collected from the same ponds were used for dilution purposes. One ml of the inoculum was transfered to a sterile petridish and pour plated. Plates were poured in duplicates and the inoculation procedures were carried out in an inoculation chamber sterilised with Ultra Violet radiations.

Incubation procedures:

For the total heterotrophs the plates were incubated at room temperature in a bell jar. Counts were made after 48 hours. For sulphate reducers plates were incubated anaerobically by using desiccator method (Rodina, 1972). First the desiccator was wiped inside with alcohol and the plates were kept in an inverted position inside the desiccator along with a burning candle. After applying vacuum grease along the rim of the lid and the desiccator was closed tightly. In the process of burning,

oxygen present inside was consumed. Alkaline pyrogallol was kept inside to ensure the removal of oxygen during incubation period. For each 100 ml capacity of jar 1 g of pyrogallol and 10 ml of 2.5 N sodium hydroxide were used(Willis,1969). Counts were made after 7 days of incubation. Black colonies were counted as sulphate reducers(PlateNo 1).

Counts were were averaged and total number of bacteria was calculated as follows:

$$\text{No.of bacteria present} = \frac{\text{No. of colonies x reciprocal of dilution}}{\text{Wt of the sample in gram}}$$

Culture of green sulphur bacteria:

Pond water was sterilised in an erlenheymer flask of 100 ml capacity. To the flask a little amount of asparagin crystals were added and the flasks were inoculated with 1 g of mud. Glass stoppers were tightly fixed and the flasks were kept near the window. Care was taken not to expose the flasks to direct Sun light. After 45 days, green colour appeared and was examined microscopically.

Stock culture maintenance:

Isolated colonies of sulfate reducers were picked and inoculated in sulfate reducing medium for further purification. Cultures were maintained in duplicates in the culture tubes des-

cribed by Pankhurst(1975).(Plate No. 2) In sulpfate reducing medium. Cultures were also maintained in screw caped test tubes in a solid medium of the same composition. (Plate No. 3). The tubes are then stored in a refrigerator, at $4^{o}C$.

Gram staining:

Huckers modified technique was used to stain the isolated strains.

Ammonium oxalate crystal violet (Huckers)

Solution - A	
Crystal violet	2 g
Ethyl alcohol (95%)	20 ml
Solution - B	
Ammolium oxalate	0.8 g
Distilled water	80 ml

Solution A and B were mixed together.

Lugol's Iodine solution (Gram's modification)

Iodine	1 g
Potassium Iodide	2 g
Distilled water	300 ml

Counter stain:

Safranin (2.5% solution in 9% ethyl alcohol)	10ml
Distilled water	100ml

Both cultures were used for staining. The heat fixed smear was stained with ammonium oxalate crystal violet for one minute

and washed in tap water for a second. The smear was then flooded with Lugol's iodine solution for one minute. After washing it in tap water the smear was decolourised in 95% ethyl alcohol and blot dried. The smear was then counter-stained for one minute in safranin solution, washed in tap water and air dried.

Gelatin utilisation test:

Utilisation of gelatin may be determined in an agar medium by adding gelatin (0.4% final concentration)to a nutrient agar, (Burnet et al., 1957). Gelatin was added to Zobell's marine agar 2216. After sterilisation the medium was poured in sufficient amount into petridishes. A single streak was made across the surface of the hardened agar. After 10 days of anaerobic incubation in desicator acid $HgCl_2$ ($HgCl_2$ 15.0 g; Con HCl 20 ml; Distilled water 100ml) was flooded onto the petridishes. A white precipitate indicated the presence of non-hydrolysed gelatin. A transparent zone around the culture indicated the presence of utilisation of gelatin.

Sodium Chloride tolerance test:

Sulphate reducing medium with different concentrations of sodium chloride were prepared. The following concentrations of 0,1,3,7, and 10 % solutions were prepared. After sterilisation all the 18 strains were inoculated in duplicates. After inoculation the original cotton plug was pushed down to the level just above the medium and plugged again with absorbant cotton without touching the original one. A small amount of pyrogallol cotton plug and 10 drops of con. sodium was added over the second carbonate solution was added. The tube was then closed with a rubber stopper tightly. The tubes were incubated at room temperature in dark. After 10 days of

incubation those tubes which were blackened were considered as positive.

Motility test:

Hanging drop preparations were made and motility was observed directly under microscope.

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RESULTS

In pond-A bottom mud was black in colour and was sandy in nature in both the sampling sites, whereas in pond-B bottom mud was black and clayey. In pond-A hydrogen sulphide smell could be noted throughout the sampling period. In pond-B during July soon after the plantation of paddy hydrogen sulphide smell in the sediment samples ceased to exist and could not be detected till the end of the sampling period. In both the ponds water samples contained more number of pigmented bacteria.

Temperature:

Temperature did not show much variation in both the ponds. In pond-A temperature varied from 28.15°C to 30°C and were recorded during the months of August and June respectively. In pond-B the temperature varied from 24.9°C to 36.25°C and were recorded during the months of August and June respectively. The highest temperature of 36.25°C recorded in June was due to a low level of water present in the pond. However such a high temperature was not recorded after June due to the plantation of paddy in the field.

Salinity:

Salinity did not show much variation in both the ponds. In pond-A it varied from 1.77‰ to 5.72‰ and were recorded during the months of August and June respectively. In pond-B salinity values varied from 1.55‰ to 3.65‰ and were recorded during the months of June and September respectively.

pH:

In pond-A there was no wide fluctuations in the pH of the sediment. It varied from 7.8 to 8.37 and were recorded during the months of June and August respectively. Water pH showed wide fluctuations. It varied from 7.25 to 8.725 and both the values were recorded during the month of July. In the pond-B the sediment pH as in pond-A did not show much fluctuations. It varied from 6.5 to 7 and were recorded during the months of July and June respectively. The maximum pH of 7 was recorded again only during the second fortnight of July. Water pH varied from 6.75 to 7.85 and were recorded during the months of June and July respectively.

Eh:

In pond-A Eh of the sediment varied from -45 mV to -167.5 mV. and were recorded during the months of September and June respectively. Eh of water varied from +75 to -60 and were recorded during the months of June and July. However, Eh of the water was positive during June and were -ve during the rest of the sampling period. In pond-B Eh of the sediment varied from -95 mV to -265 mV and were recorded during the month of July. Water Eh as in the case of pond-A was +ve during June and July but -ve during the rest of the period of study. It varied from -10mV to +77.5 mV and were recorded during the months of September and June respectively.

Dissolved Oxygen:

Dissolved oxygen content in pond-A varied from 4.115 to 6.35ml/l and were recorded during the months of september and July. In pond-B it varied from 2.24 to 5.15 ml/l and were recorded during the months of September and July. Dissolved oxygen content showed same type of variation in both the ponds. Maximum values were recorded during the month of July. After July dissolved oxygen content decreased in both the ponds. However, generally, dissolved oxygen content was more in pond-A than in pond-B, during the entire study period.

Bacterial Parameters:

Total Heterotrophs:

Total heterotrophs showed a similar trend of variation in both the ponds. In pond-A total heterotrophic counts in the sediments varied from 11 x 10^5/g mud to 23 x 10^6/g mud and were recorded during the months of August and June respectively. Total heterotrophic count in water varied from 15.5 x 10^4/ml to 21.0 x 10^5/ml and were recorded during the months of August and June. In pond-B total heterotrophic population in mud varied from 15 x 10^5/g mud to 29.5 x 10^6/g mud and were recorded during the months of August and June. The maximum value of 29.5 x 10^6/g mud was observed during the first fortnight of July also. Total heterotrophic counts in water varied from 13 x 10^4 to 23 x 10^5/ml and were recorded during the months of September and June

A sudden decrease in bacterial populations both in mud and water, was noted in August in both the ponds.

Sulphate reducers:

Sulphate reducers also showed the same trend of variation as total heterotrophs, and the trend was similar in both the ponds. In pond-A the counts varied from 2.45×10^2/g mud to 6.6×10^2/g mud. The maximum number was observed during the month of June and minimum value of 2.45×10^2/g mud was observed during the months of July, August and September. In pond-B sulphate reducers varied from 2.45×10^2/g mud to 20.8×10^2/g mud and were recorded during the months of August and June respectively. A sudden decrease in the bacterial population was noted as in the case of total heterotrophs in the month of July.

Statistical Analysis

Multiple regression analysis was carried out to find out the relationship between different environmental parameters and sulphate reducers. In both the ecosystems, salinity, sediment Eh, and total heterotrophic populations both in the mud and water were found to have correlation with sulphate reducers. Standard partial regression analysis was done to find out the degree of correlation of each parameter with sulphate reducers. It was found that, in pond-A Sulphate reducers showed correlation with the environmental parameters in the order, Eh of the sediment, salinity, total heterotrophic populations

in sediments and total heterotrophic population in water. In pond-B, however, the following order was found: Total heterotrophic population in water, total heterotrophic population in sediments, sediment Eh and salinity. The results are given in the table No. IV .

Multiple regression equations were fitted, and are:

Ecosystem A

$$y = -27.3146 - 0.4641x_1 + 10.3392x_2 - 1.877x_3 + 0.6041x_4$$

$$aR^2 = 86.06\ \%$$

where x_1 = sediment Eh, x_2 = salinity, x_3 = total heterotrophs in mud and x_4 = total heterotrophs in water.

Ecosystem B

$$y = 13.7395 - 0.2753x_1 - 5.2907x_2 - 4.2666x_3 + 10.9125x_4$$

$$aR^2 = 74.8\ \%$$

where x_1 = sediment Eh, x_2 = salinity, x_3 = total heterotrophs in mud and x_4 = total heterotrophs in water.

Biochemical tests:

Microscopical examination of cultures of green sulfur bacteria showed the presence of Gram negative, non motile, spherical cells. Cells were bound together in a mucous-like substance.

Altogether 18 isolates of sulphate reducers were selected and biochemical tests were conducted. Results showed that all the isolates were Gram negative and motile. Their morphology varied from small curved

rods to straight rods. All the isolates produced H_2S. Sodium chloride tolerence test showed that they failed to grow in 7 % and 10 % solutions. All the isolates grew in the NaCl concentrations 0 %, 1 %, and 3 %. The results of the biochemical tests were compared with the taxonomic scheme given in the Bergey's Manual of determinative bacteriology (1974). It was found that the species involved in the process of sulphate reduction in ponds-A and B were Desulfovibrio desulfuricans and Desulfovibrio aestuarii. (Plate Nos 4&5)

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Table No: I. BACTERIOLOGICAL AND ENVIRONMENTAL PARAMETERS OF POND - A

Month	Water temperature °C	Sediment pH	Water pH	Sediment Eh mV	Water Eh mV	Dissolved oxygen ml/l	Salinity °/oo	Bacterial count		
								THS No./gmud	THW No./ml	SRB No./gmud
JUN	30.0	7.95	8.5	-122.0	+66	4.65	5.721	23.0×10^{6}	15.0×10^{5}	6.60×10^{2}
	28.25	7.8	8.5	-119.5	+75	4.75	3.34	20.5×10^{6}	21.0×10^{5}	5.50×10^{2}
JUL	29.80	8.0	8.25	-167.5	-60	6.25	2.07	20.0×10^{6}	19.0×10^{5}	5.80×10^{2}
	28.30	8.05	8.725	- 50.0	-15	6.35	2.30	7.0×10^{6}	14.0×10^{5}	2.45×10^{2}
AUG	28.70	8.05	7.25	- 75.0	-15	6.0	2.10	14.0×10^{5}	16.0×10^{4}	2.60×10^{2}
	29.00	8.30	8.50	- 85.0	-25	5.0	1.77	$11\times0\times10^{5}$	15.5×10^{4}	2.45×10^{2}
SEP	28.15	8.275	8.45	- 55.0	-25	4.405	2.61	33.5×10^{5}	26.0×10^{4}	2.45×10^{2}
	29.09	8.275	8.725	- 45	-25	4.115	3.24	33.0×10^{4}	27.5×10^{4}	3.20×10^{2}

THS Total heterotrophs in sediment

THW Total heterotrophs in water

SRB Sulphate reducing bacteria

Table No: II. **BACTERIOLOGICAL AND ENVIRONMENTAL PARAMETERS OF POND - B.**

Month	Water temperature °C	Sediment pH	Water pH	Sediment Eh mV	Water Eh mV	Dissolved oxygen ml/l	Salinity °/oo	Bacterial count THS No./gmud	THW No./ml	SRB No./gmud
JUN	36.25	7.00	6.75	-205	+48.2	4.7	2.856	20.0×10^6	20.0×10^5	20.8×10^2
	28.10	6.75	7.75	-205	+77.5	4.85	3.57	29.5×10^6	23.0×10^5	15.2×10^2
JUL	28.60	6.75	7.75	-265	+63.0	4.65	1.55	29.5×10^6	18.5×10^5	16.0×10^2
	26.15	6.57	7.85	- 95	+ 3.0	5.15	2.0	26.5×10^6	12.5×10^5	5.5×10^2
AUG	26.05	7.0	7.35	-120	-11.5	4.95	3.0	16.0×10^5	21.5×10^5	5.2×10^2
	24.90	6.95	7.35	-130	-15.0	3.8	3.05	15.0×10^5	21.5×10^5	2.45×10^2
SEP	25.10	6.95	6.825	-115	-15.0	3.5	2.75	22.5×10^5	18.0×10^4	2.45×10^2
	28.00	6.725	7.475	-155	-10.0	2.24	3.65	24.0×10^5	18.0×10^4	8.1×10^2

THS Total heterotrophs in sediment

THW Total heterotrophs in water

SRB Sulphate reducing bacteria

Table No.III

TABLE SHOWING THE RESULTS OF THE TESTSFOR IDENTIFICATION OF SULPHATE REDUCERS

Tests	Percentage of +ve result	Percentage of -ve result
Gram's staining	100	0
Motility	100	0
Gelatin utilisation	33	67
NaCl tolerence		
0 %	100	0
1 %	100	0
3%%	100	0
7 %	0	100
10 %	0	100
Aerobic growth	0	100
H_2S production	100	0

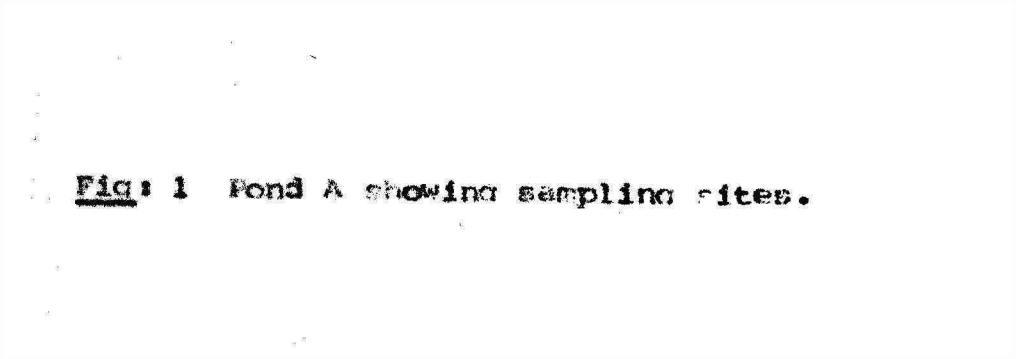


Fig: 1 Pond A showing sampling sites.

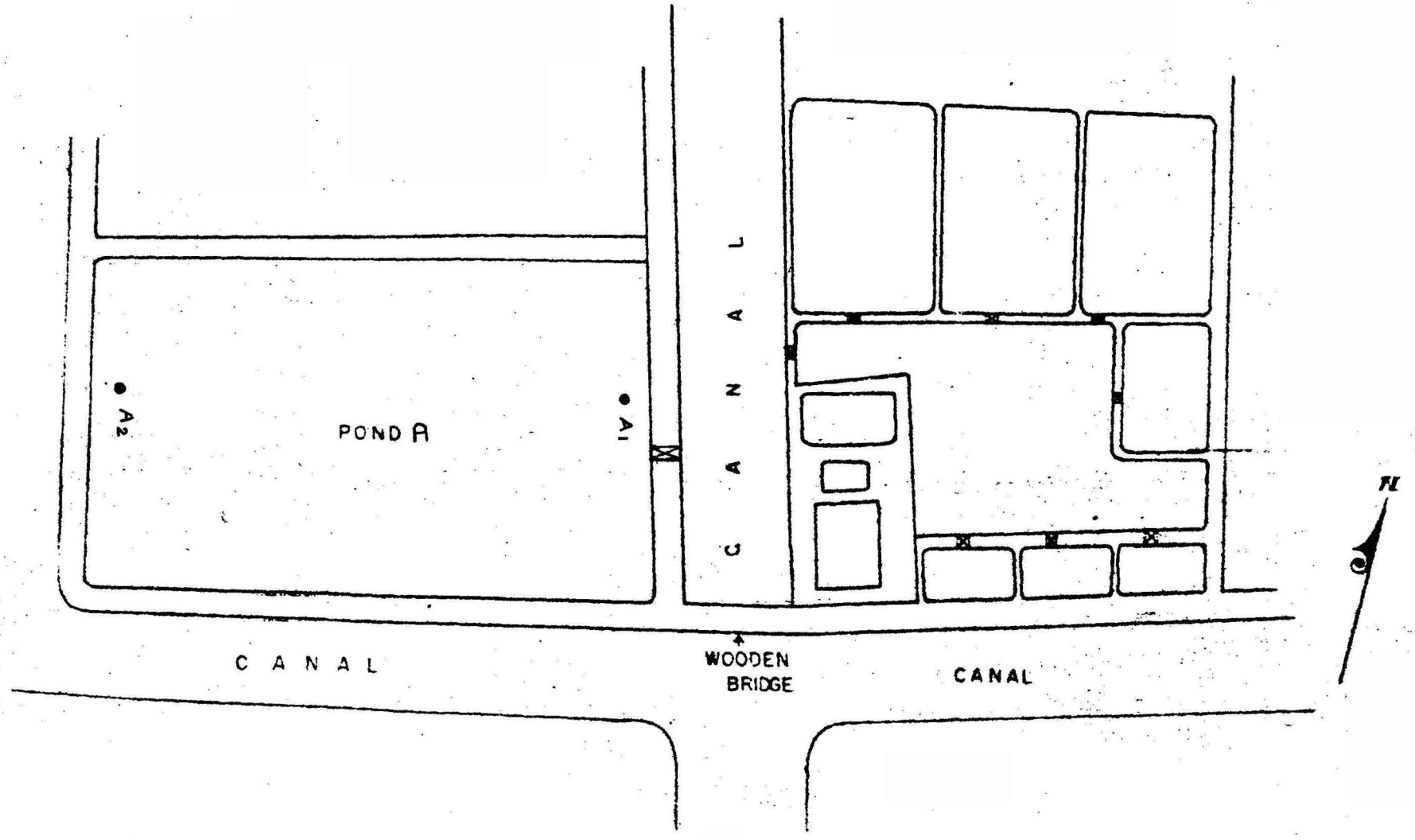
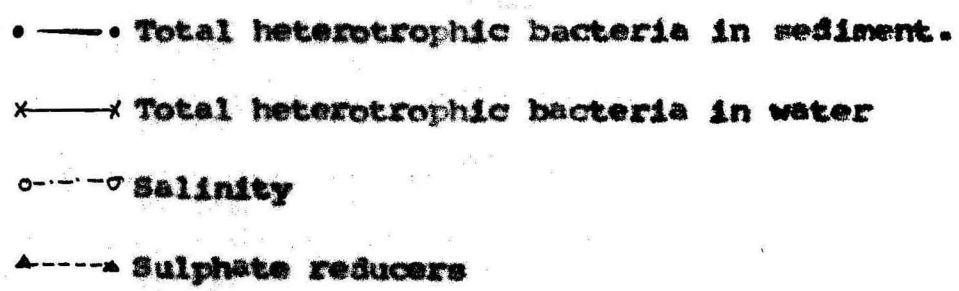

A2
POND A
A1
CANAL
CANAL
WOODEN
BRIDGE
CANAL
N

Fig: 2 summary of variations in salinity and bacterial populations. (Pond - A)



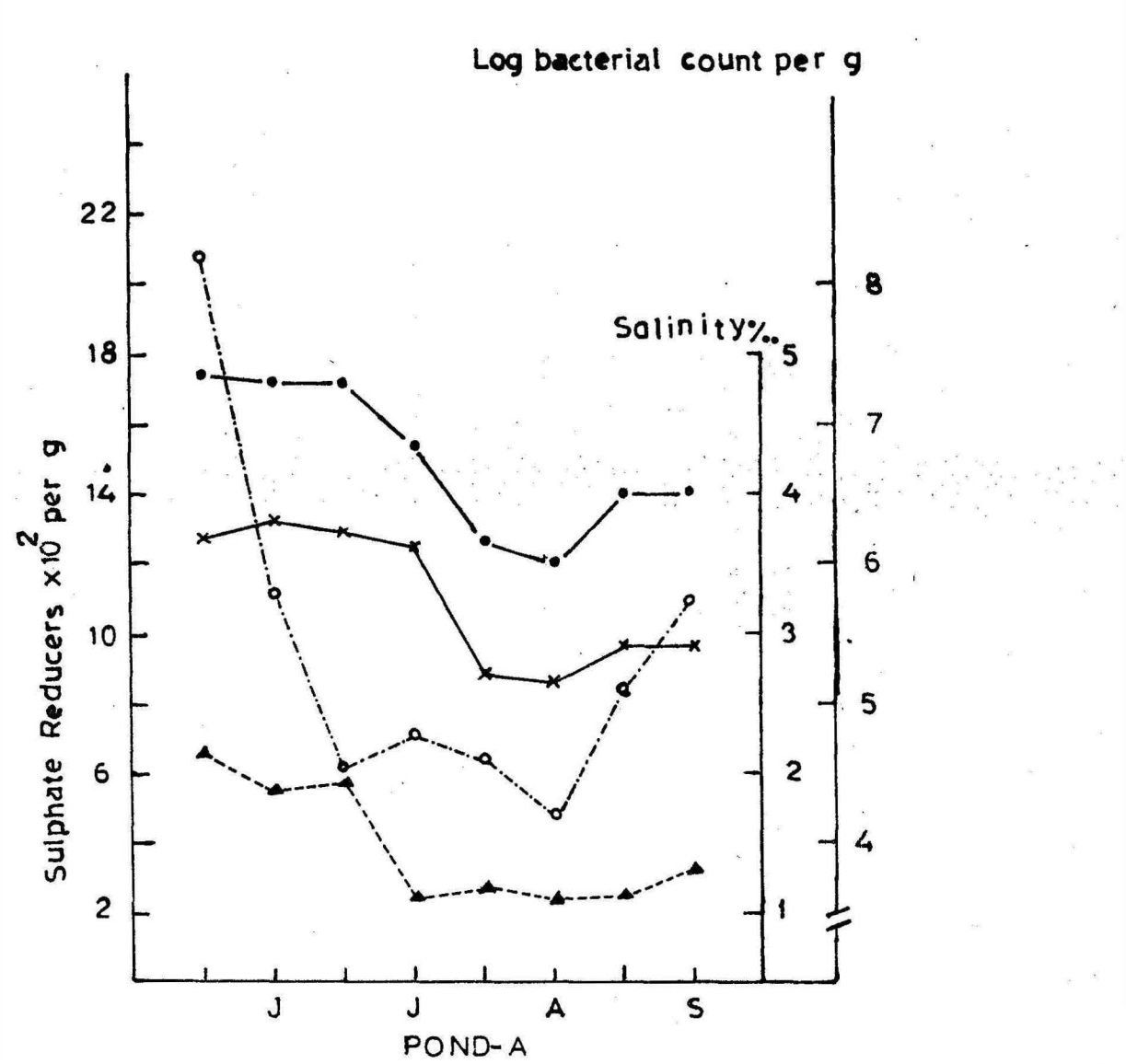

Log bacterial count per g
Salinity%
Sulphate Reducers x10² per g
22
18
14
10
6
2
8
7
6
5
4
3
2
1
5
4
J
J
A
S
POND-A

Fig : 3 Summary of variations in salinity and bacterial populations. (Pond-B)

•——• Total heterotrophic bacteria in sediment.

×——× Total heterotrophic bacteria in water.

○---○ Salinity.

▲---▲ Sulphate reducers.

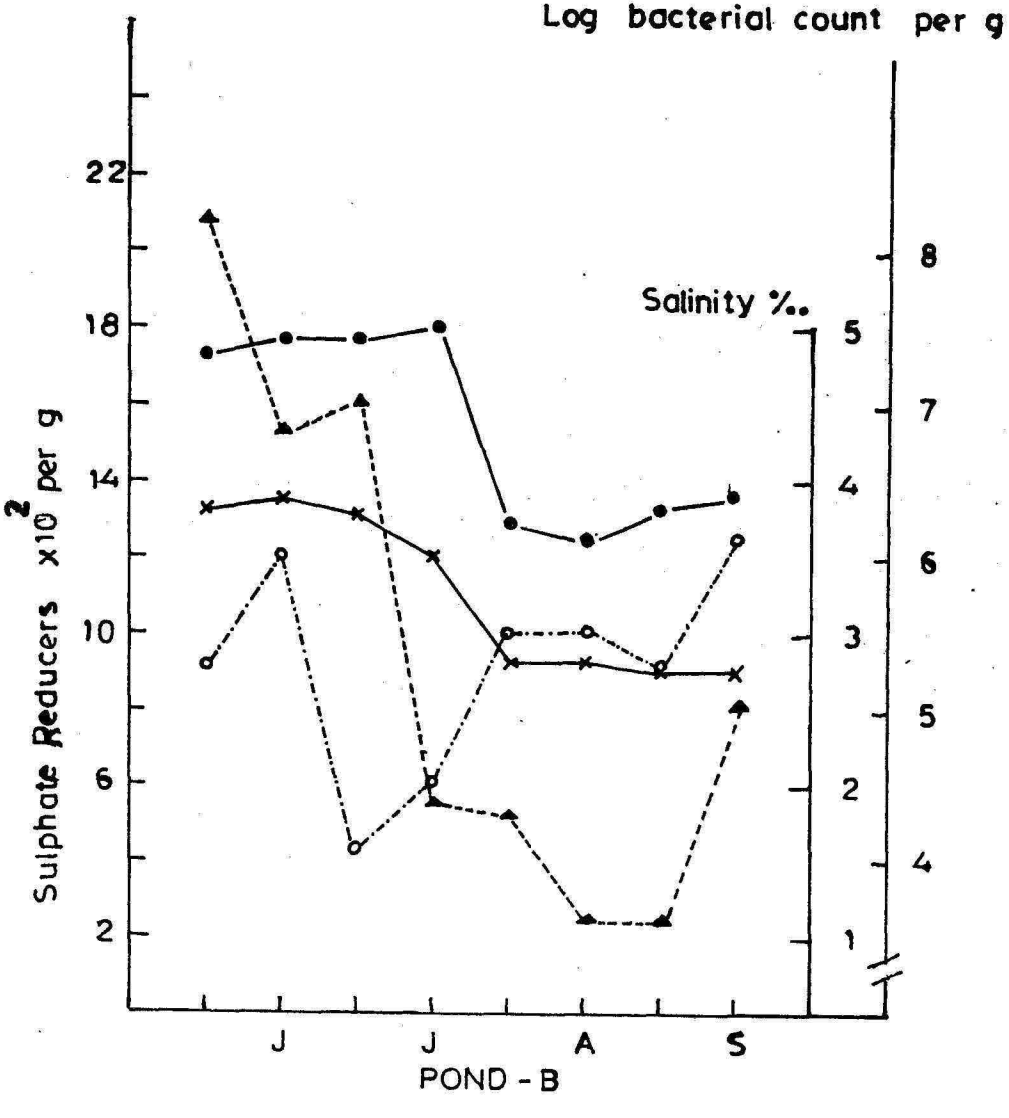

Log bacterial count per g
Sulphate Reducers x10 per g
Salinity ‰
22
18
14
10
6
2
8
7
6
5
4
3
2
1
5
4
J
J
A
S
POND - B

Fig: 4 Summary of variations in temperature and oxygen.

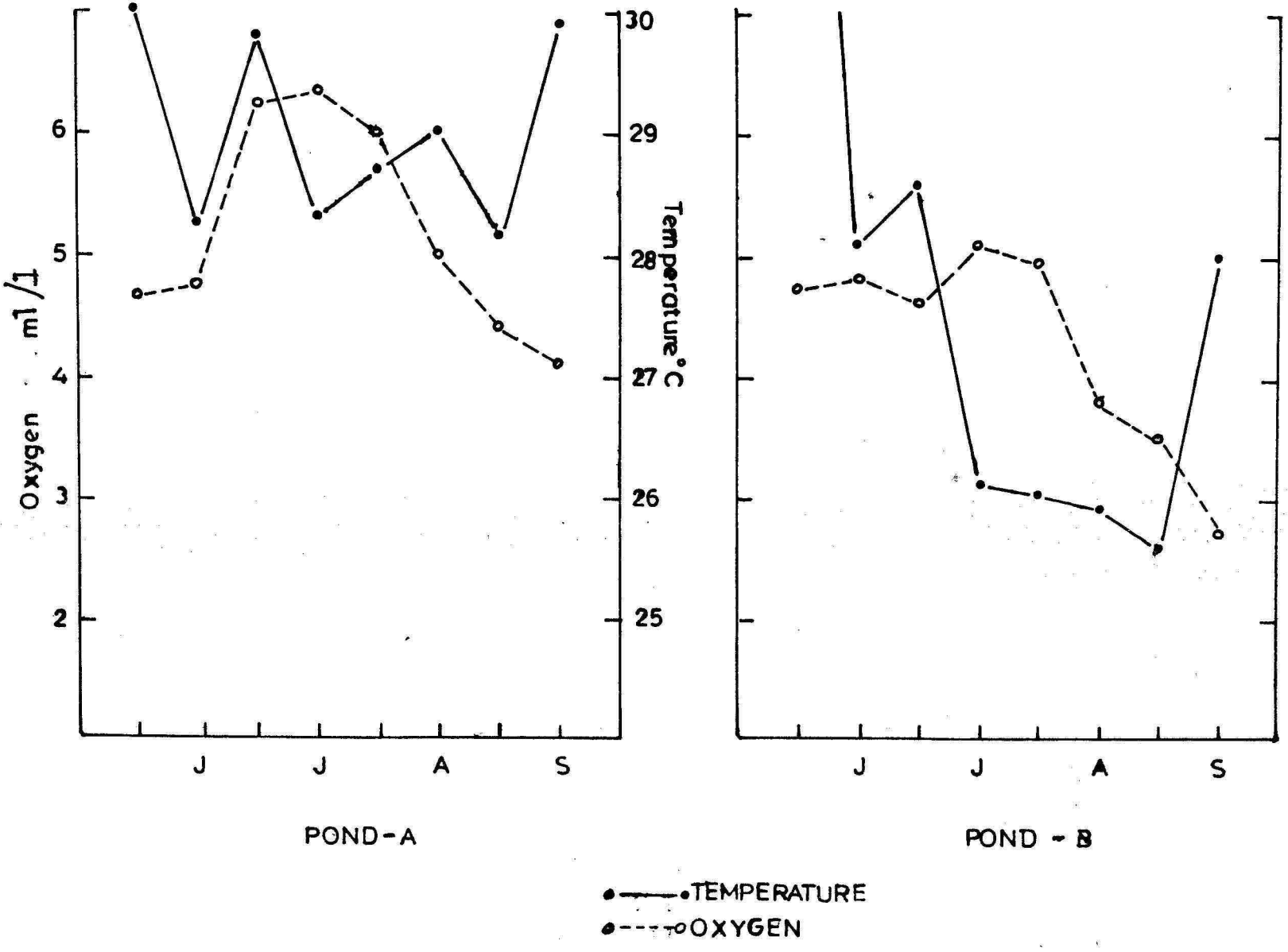

Oxygen . ml/l
Temperature °C
6
5
4
3
2
30
29
28
27
26
25
J
J
A
S
POND-A
POND - B
TEMPERATURE
OXYGEN

Fig: 5 Summary of variations in Redox Potential.

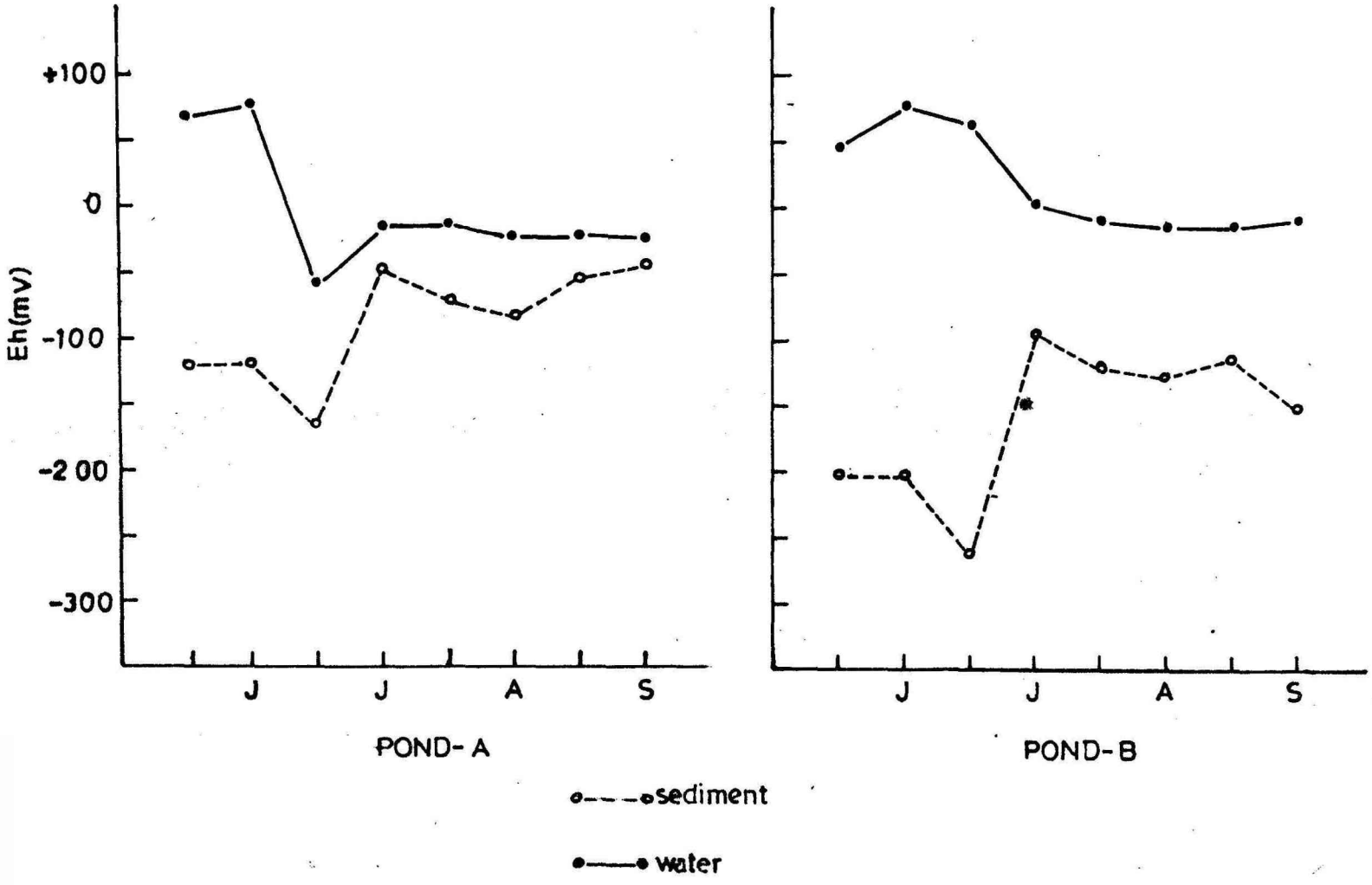

+100
0
-100
-200
-300
Eh(mV)
J
J
A
S
POND- A
POND- B
sediment
water

Fig 6: Summary of variations in pN.

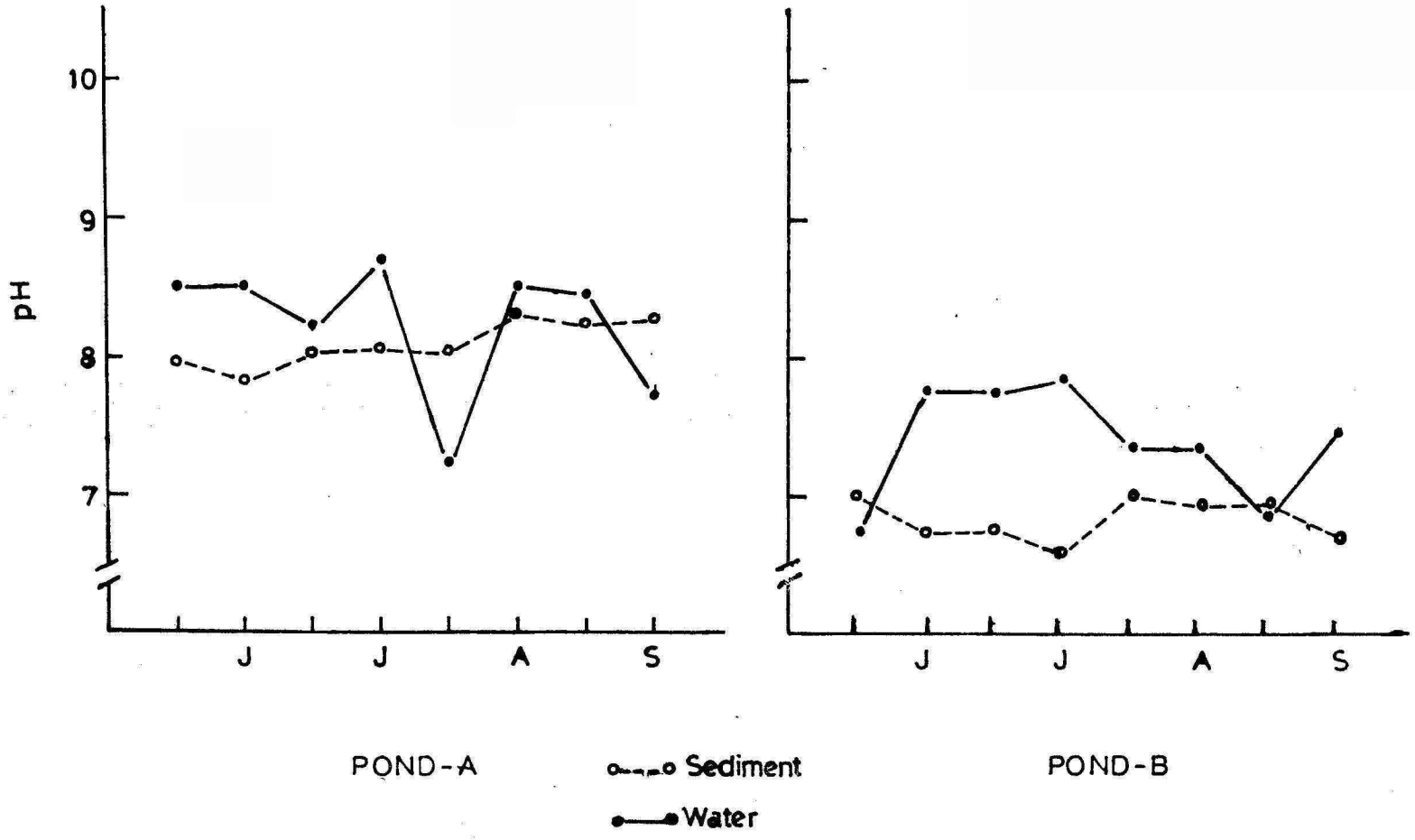

10
9
8
7
pH
J
J
A
S
J
J
A
S
POND-A
Sediment
Water
POND-B

Plate 1

Sulphate reducing bacteria in lead acetate agar.

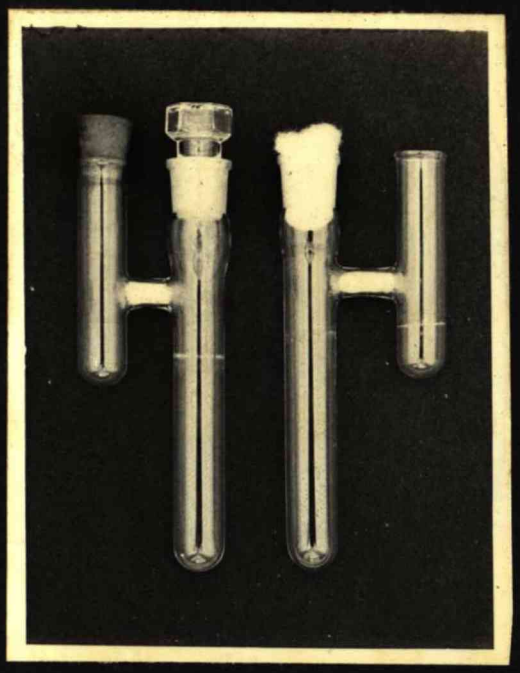


Plate 2

Pankhurst's culture tubes.

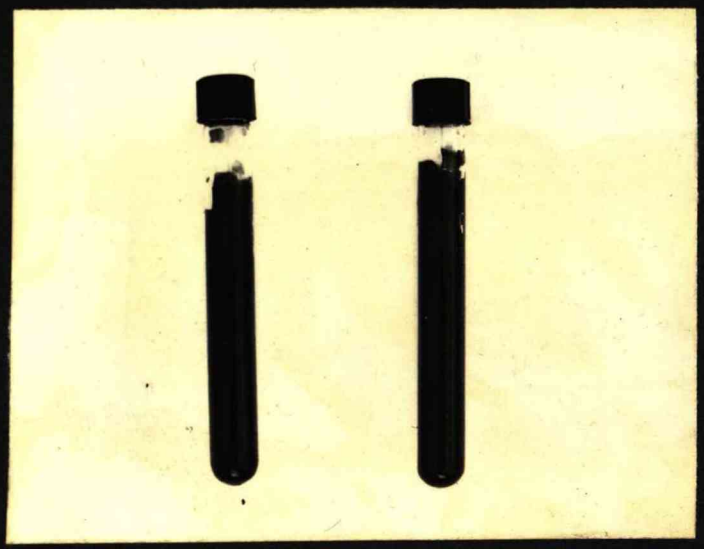
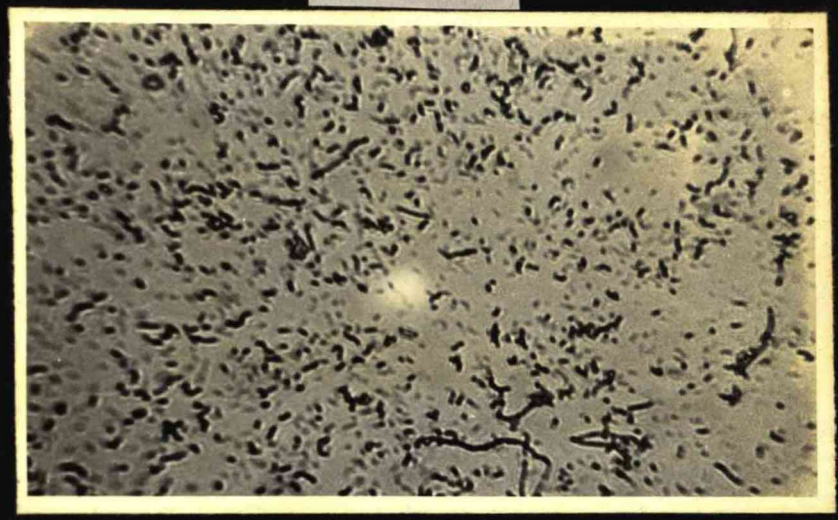


Plate 3

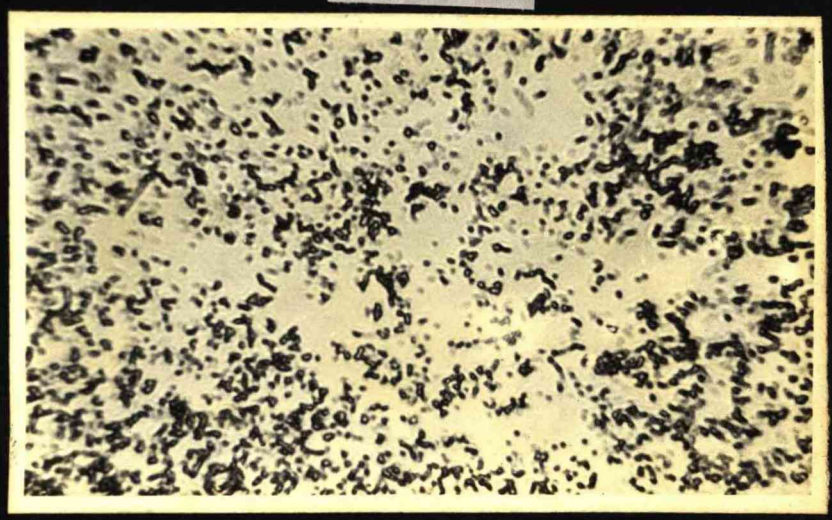
Sulphate reducing bacteria in screw capped test tubes.

Plate 4



Desulfovibrio desulfuricans

Plate 5



Desulfovibrio aestuarii

Plate 6

Green sulfurbacteria

DISCUSSION

In the present study variation in sulphate reducing bacteria along with other bacterial and environmental parameters were monitered for a period of four months from one perennial pond (pond-A) and from a pokkali field (pond-B).

During the entire sampling period in both the ponds bottom sediments were black in colour due to the precipitation of ferrous sulphide caused by sulphate reduction. Bunker(1936) attributed the blackening of mud entirely to the action of sulphate reducers in marine and estuarine environments.

In pond-A during the period of investigationhydrogen sulphide swell was noticed in core samples. In pond-B however hydrogen sulphide ceased to exist soon after paddy plantation. In pond-B before starting paddy cultivationwater was completely drained and the bottom soil was ploughed, tilted and exposed to Sun. This caused a complete aeration and an increase in temperature of the soil. The complete aeration and increased temperature of the soil might have oxidised the hydrogen sulphide present in the soil and this might be the reason for the ceasation of H_2S during the period of investigation. Several investigators reported that the release of hydrogen sulphide from anoxic sediments due to high temperature caused oxygen demand in the overlying water due to the chemical oxidation of hydrogen sulphide in presence of oxygen.(Poole et al.,1977; Parkes et al., 1979; Parkes and Poole,1980.). Parkes and Poole (1980) recorded a

decrease in sulphide due to sediment disturbance caused by storms.

In the present investigation maximum number of sulphate reducing bacteria recorded from pokkali field is 2.08×10^3/g mud. Sethunathan *et al.*, (1981) reported a number of 17.7×10^4/g mud in pokkali field. As this study was carried out during monsoon season, the considerable decrease in sulphate reducers might have happened during the premonsoon period and might have been caused by the combined effect of high temperature during the summer, aeration of the soil during the preparation of the field for paddy cultivation and the toxicity of hydrogen sulphide to bacterial populations. Parkes and Poole(1980) also observed a decrease in sulphate reducing bacterial population during summer due to the high temperature and toxicity of hydrogen sulphide in the River Don estuary. In pond-B temperature started decreasing from June onwards due to monsoonand showed a positive relationship with sulphate reducers. ($r = .7960$). Nedwell and Flood Gate(1972) reported that sulphide formation through sulphate reduction was inhibited at low temperature on the basis of the lab experiments.

During the study period salinity showed more fluctuations in pond-A than in pond-B. In pond-A salinity showed a positive relationship and was found to be the prime factor influencing the sulphate reducers(Table No. IV). It may be attributed to the constant exchange of water due to the tidal fluctuation. In pond-B salinity showed only a little fluctuation and had a

lesser effect on sulphate reducers as there was no water exchange in the pond. Similar type of observation were made by many workers. An extremely close relationship between sulphate reducers and salinity was noted by Parkes et al., (1979) and Parkes and Poole(1980). Tezuka(1970) recorded more number of sulphate reducers from brakish water and marine environments than in fresh water environments. It may be due to the availability of sulphate in sea water. (Tezuka, 1979; Parkes and Poole, 1980).

Sediment pH in both the ponds did not show much fluctuations. Maximum and minimum values recorded in the present study were 6.5 and 8.3 (Table No.I&II). Zobell (1958) reported that the range of pH values for suphate reducers as 6.4 to 9.5 in marine bottom deposits. Cultures of Desulfovibrio desulfuricans examined by Starkey and Wight (1945) grew over the range of pH 5.5 to 8.5 and the optimum was near 7. Zobell (1958) reported that pH tolerence of sulphate reducers both in the natural environments and in the contsolled laboratory conditionsis affected by Eh of the environment. Poole et al.,(1977) reported the occurence of sulphate reducers in the pH values ranged between 6.7 and 7.1. Parkes and Poole(1980) recorded the occurence of sulphate reducers between the pH range of 6.8 to 7.04. In the present study the sediment pH values in both the ponds did not show any relationship with sulphate reducers within the range of pH so far reported. Water pH in both the ponds was varying and did not show any relationship with sulphate reducers.

Eh values have positive relationship with sulphate reducersin both the ponds(Table No I&II). Zobell(1958) reported that the reaction of marine bottom deposits in which sulphate reducers occur ranges from Eh +350 to -500 mV. Desulfovibrio sp. isolated with micropipette have developed in nutrient medium only when the initial Eh was 0 to -100 mV (Zobell,1958). Starkey and Wight(1945) reported that the culture media used for the culture of Desulfovibrio desulfuricans was initially 300 mV to 400 mV and dropped to -200 to -300 during growth. Eh values recorded when sulphate reduction was maximum from estuarine environments were -176 mV and -160 mV(Parkes et al.,1979) and Parkes and Poole, 1980). A close relationship between change in Eh values and sulphate reduction was noticed by Parkes and Poole(1980). In the present study minimum Eh values recorded in pond-A is -167.5 and is within the range which is controlled by sulphate reduction. A value of -265 mV was recorded in pond-B. As there could not be any hydrogen sulphide detected in pond-B during the period of investigation, this low value of Eh might not have been caused by sulphate reduction and may be due to the clyey nature of the soil and the reducing activity of sedimentary bacteria(Berner,1963), particularly those bacteria which release sulphur from organic materials.

Oxygen depletion due to hydrogen sulphide production in water bodies has been reported by Poole et al.,(1977) and Parkes et al.,1979). Poole et al.,(1977) noted that the sediments and water

column were anoxic during summer months due to the presence of hydrogen sulphide. The depletion of oxygen is mainly attributed to the chemical oxidation of sulphide(Poole et al., 1977). In the present study dissolved oxygen content did not show any relationship with sulphate reducers but decreased during the course of investigation in both pond-A and pond-B (Figure 4).

In the present study sulphate reducers showed a positive relationship with total heterotrophic populations (Fig.2&3). Poole et al., (1977), Parkes and Poole (1979 and 1980) reported similar trend of variation in different groups of bacteria and concluded that these bacterial populations were under the influence of a common factor. In the present study also a sudden decrease in population of sulphate reducing bacteria and total heterotrophs both in water and mud was noticed and may have been due to the frequent, heavy rains occured during the sampling period. However no sudden change in any environmental parameters was noticed.

As sulphate reducers can utilise only a limited group of organic compounds (LeGall & Postgate, 1973), it is proposed that they must rely on the metabolic byproducts of other heterotrophic bacteria(Tezuka, 1966 ; Poole et al., 1977). In upper L'Etang estuary, Canada which is polluted with paper mill effluent, it was found out that the sulphate reducers rely on the anaerobic cellulolytic bacterial population or anaerobic heterotrophic bacteria associated with that process (Poole

et al., 1977). In the pokkali field as the paddy stumps are allowed to disintegrate in the field itself, it considerably increases the organic content of the soil. Especially the cellulose content of the soil is increased Thus a same type of bacterial population as in the upper L'Etang estuarymay occur in the pokkali fields also.

In the present study a relatively high number of total heterotrophic bacteria in the sediment were recorded in the pond-B (Average value for the study period was 14.15×10^6/g mud) than in the pond-A(Average value for the study period was 9.95×10^6/g mud). The same trend was closely followed by the sulphate reducers also. Population of sulphate reducers in the pond-B was almost double the amount of sulphate reducers in the pond-A (Average values were 4.47×10^2 and 3.80×10^2/g mud respectively). This observation supports the view of the earliar workers that there is a nutritional relationship between sulphate reducers and other heterotrophs. Tezuka (1966) reported such a nutritional commensalism between sulphate reducers and other heterotrophic bacteria. Ibrahim et al.,(1981) proposed such a relationship between sulphate reducers and other organotrophs.

Bacterial sulphate reduction is the penultimate stage of gross organic pollution. The development of anoxic conditions and the appearance of sulphide in a culture ecosystems is highly toxic to the culture animals, whose death may inturn deteriorate the water quality and augment the gross pollution. So, proper

measures are to be taken for the control and removal of hydrogen sulphide from culture ecosystems.

Shigueno (1975) found out that the level of hydrogen sulphide could be decreased in culture ponds by the application of ferrous oxide without any harm to the cultured animals. Sethunathan et al., (1981) reported that the application of Hexachloro cyclo hexane (HCH) and Benomyl at a level of 100 ppm in the pokkali fields inhibited the sulphate reduction to sulphide and reduced the population of sulphate reducers from an initial value of 1.7×10^4/g mud to a final value of 0.14×10^4 and 0.11×10^4/g mud respectively.

More work should be done in this regard to have a better understanding about the factors involved, and processes working within the ecosystem in the formation of hydrogen sulphide through sulphate reduction.

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SUMMARY

1. Studies on the short term variations in the sulphate reducing bacterial populations were monitored for a period of four months from June to september along with some of the bacterial and environmental parameters in two different prawn culture ecosystems, a pokkali field and a perennial pond.

2. In pond-A water level was varying and the constant exchange of water was effected by tides. In pond-B, water was stagnant and the pond was shallow. Paddy was under cultivation during the period of study.

3. In the pokkali field bacterial populations were more than in Pond -A.

4. Sediment Eh, salinity and total heterotrophic populations were found to have correlations with sulphate reducers. In Pond-A they were in the order of sediment Eh, salinity and total heterotrophic populations. In pond-B however the order was total heterotrophic populations, sediment Eh and salinity. In the pokkali field temperature was also found to have positive relationship with sulphate reducers.

5. Both sulphate reducers and total heterotrophs showed a same trend of variations during the study period.

6. sediment pH did not show much variations. Water pH and water Eh were varying and did not show any relation ship with sulphate reducers in both the ponds.

7. Dissolved oxygen content increased during the month of july and decreased during the rest of the period of study in both the ponds and did not show any relationship with sulphate reducers.

8. In pond-A hydrogen sulphide smell could be noted in core samples throughout the period of study but it ceased to exist in pond-B soon after the plantation of rice.

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